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Molecular identification of zeaxanthin epoxidase of *Nicotiana plumbaginifolia*, a gene involved in abscisic acid biosynthesis and corresponding to the *ABA* locus of *Arabidopsis thaliana*

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Abscisic acid (ABA) is a plant hormone which plays an important role in seed development and dormancy and in plant response to environmental stresses. An ABA-deficient mutant of *Nicotiana plumbaginifolia*, *aba2*, was isolated by transposon tagging using the maize *Activator* transposon. The *aba2* mutant exhibits precocious seed germination and a severe wilty phenotype. The mutant is impaired in the first step of the ABA biosynthesis pathway, the zeaxanthin epoxidation reaction. *ABA2* cDNA is able to complement *N.plumbaginifolia aba2* and *Arabidopsis thaliana aba* mutations indicating that these mutants are homologous. *ABA2* cDNA encodes a chloroplast-imported protein of 72.5 kDa, sharing similarities with different monooxigenases and oxidases of bacterial origin and having an ADP-binding fold and an FAD-binding domain. ABA2 protein, produced in *Escherichia coli*, exhibits *in vitro* zeaxanthin epoxidase activity. This is the first report of the isolation of a gene of the ABA biosynthetic pathway. The molecular identification of *ABA2* opens the possibility to study the regulation of ABA biosynthesis and its cellular location.

Keywords: abscisic acid/*Arabidopsis thaliana*/carotenoid/*Nicotiana plumbaginifolia*/transposable element

Introduction

Abscisic acid (ABA) is a naturally occurring plant hormone that was identified in the 1960s. ABA is present in all higher plants as well as in some mosses, green algae, fungi and bacteria. In higher plants, ABA has been implicated in the control of a wide range of essential physiological processes including seed development, germination and plant tolerance to different stresses (reviewed by Zeevaart and Creelman, 1988). Despite its capital importance, ABA is probably one of the less well characterized plant hormones. The majority of information concerning ABA physiology and its functional significance in plants has been derived from the analysis of the effects of exogenous applied ABA on plants and from the characterization of ABA-biosynthetic mutants. ABA-deficient mutants, identified in a variety of plant species, display various abnormalities including precocious germination and a strong tendency to wilt, and can be restored to a wild-type phenotype by an exogenous supply of ABA. ABA-deficient mutants have also contributed extensively to the clarification of the biosynthetic pathway of the hormone (reviewed by Giraudat *et al.*, 1994).

ABA is a sesquiterpenoid, the precursor of which is mevalonic acid. Phytopathogenic fungi synthesize ABA by a direct pathway (the so called C_{15} pathway) from farnesyl pyrophosphate, whereas ABA biosynthesis in higher plants likely proceeds via an indirect pathway which involves C_{40} carotenoid precursors (reviewed by Zeevaart and Creelman, 1988; Walton and Li, 1995). In plants, ABA is synthesized from xanthophylls (oxygenated carotenoids), e.g. zeaxanthin, violaxanthin and neoxanthin (Li and Walton, 1990; Parry *et al.*, 1990). Studies of the biochemical basis of genetic lesions in several mutants have confirmed the occurrence of the indirect pathway in plants. Some of the *viviparous* mutants of maize (*vp2*, *vp5*, *vp7* and *vp9*) are blocked in the early stages of carotenoid biosynthesis. The abnormal complement of carotenoids appears to result in both photobleaching and ABA deficiency (Moore and Smith, 1985; summarized by Taylor, 1991). The *aba* mutant of *Arabidopsis thaliana* (Koornneef *et al.*, 1982) is impaired in the epoxidation of zeaxanthin into antheraxanthin and violaxanthin, which is now considered as the first step in the biosynthesis of ABA (Duckham *et al.*, 1991; Rock and Zeevaart, 1991). Several mutants isolated in tomato (*flacca* and *sitiens*), potato (*droopy*), barley (*nar2a*) and *Nicotiana plumbaginifolia* (*aba1*) are impaired in the last step of the pathway: the oxidation of ABA-aldehyde to ABA (reviewed by Taylor, 1991). For the intervening steps of the ABA biosynthetic pathway, only two possible candidates exist: the *wilty* mutant of pea (Duckham *et al.*, 1989) and the *notabilis* mutant of tomato (Parry *et al.*, 1988). These mutants have not been sufficiently characterized to identify their biochemical lesions (Taylor, 1991).

Despite considerable progress in the elucidation of the ABA biosynthesis pathway, no gene of this pathway has been cloned to date. Isolation of such genes will facilitate several avenues of research: a better understanding of the biosynthesis pathway itself and its cellular location, and the genetic manipulation of ABA level in transgenic plants, in order to study the physiological role of ABA in plant development, seed dormancy and maturation, and in stress tolerance.

We describe here the genetic and biochemical character-

ization of the *aba2* mutant impaired in ABA biosynthesis, which has been isolated after *Ac* insertional mutagenesis in *N.plumbaginifolia*. We present also evidence that *ABA2* cDNA encodes a chloroplastic protein, the zeaxanthin epoxidase. Finally, complementation of *N.plumbaginifolia aba2* and *A.thaliana aba* mutations by *ABA2* cDNA indicates that these two loci are homologous.

Results

Isolation of an ABA-deficient mutant in a N.plumbaginifolia line carrying the Ac transposable element

The maize transposable element *Ac*, inserted in the leader sequence of a kanamycin resistance marker gene (Baker *et al.*, 1987), was introduced via *Agrobacterium*-mediated transformation into *N.plumbaginifolia*, giving rise to primary transformants designated R_0 (Marion-Poll *et al.*, 1993). *Ac* transposition was monitored in their progeny (R_1) by using the kanamycin resistance excision marker. This allowed the selection of 1000 kanamycin resistant R_1 plants. They were grown to maturity and their progeny sown *in vitro*. One of them (the R_1 plant number AA67) segregated for an early germination and wilty phenotype. Out of 658 seeds, 164 were found to germinate precociously. This frequency fits very well with a 3:1 hypothesis ($\chi^2 = 0.002$; $P > 0.95$) suggesting that the early germination phenotype is due to a single recessive nuclear mutation. Freshly harvested seeds of the mutant germinated in 4–6 days, whereas wild-type seeds required 10–15 days (Figure 1A). Young mutant plantlets were extremely sensitive to hydric stress (Figure 1D and E) probably because they fail to close their stomata (Figure 1B and C). Mutant leaves lost 48% of their fresh weight (FW) within 60 min under a stream of air whereas in the same period, wild-type leaves lost only 11% FW (Figure 1F). Therefore mutant plants were grown in an environment of 70–90% relative humidity. The phenotype was consistent with ABA-deficient or ABA-insensitive mutants. To discriminate between these possibilities, young mutant plants were treated with a daily spray of a 40 μM ABA solution for 2 weeks. This treatment restored the wild-type transpiration rate when assayed on detached leaves (data not shown). Grafting of mutant plants onto tobacco wild-type stocks also restored a wild-type phenotype. Since the mutant was indeed sensitive to ABA, it was concluded that it was likely to be ABA deficient. The mutant was then crossed to *aba1*, an ABA-deficient mutant previously isolated in *N.plumbaginifolia* (Bitoun *et al.*, 1990; Blonstein *et al.*, 1991; Rousselin *et al.*, 1992) and shown to complement the *aba1* mutation. The new locus isolated was therefore called *ABA2*.

The aba2 mutation is due to an Ac insertion

Cosegregation of the aba2 mutation with an Ac-hybridizing band. DNAs from mutant and wild-type progeny of plant AA67 were analyzed by digestion with different enzymes and hybridized with probes A and B, which are *Ac* internal fragments (Figure 2B). In an *Eco*RI digest, probed with A (Figure 2A), the primary transformant (R_0) presents a single *Ac*-hybridizing band of 3 kb, corresponding to the *Ac* location within the T-DNA. In the R_1 AA67 plant, two new *Ac* insertions (4.5 and 2.6 kb bands) are revealed by the same probe. The 2.6 kb band is unlinked to the *aba2* mutation. The presence of the 4.5 kb band was investigated in 39 R_2 plants: 13 of them exhibited the mutant phenotype whereas 26 showed the wild-type phenotype. All the mutant plants had the 4.5 kb *Ac*-hybridizing band. Eight of the 26 phenotypically-normal plants did not exhibit the 4.5 kb band and had wild-type progeny. The other 18 plants segregated in their progeny 25% plantlets with the mutant phenotype and all but one had the 4.5 kb band. The proportion of plants with the 4.5 kb *Ac*-hybridizing band among the wild-type progeny (two-thirds) is in accordance with the proportion of heterozygous plants, suggesting that the mutation was indeed linked to this *Ac* band.

The aba2 mutation is unstable. The absence of the 4.5 kb *Ac*-hybridizing band in one of the heterozygous plants might be explained by an imprecise excision event that did not restore the functionality of the gene. In order to test this possibility, the genomic *Kpn*I fragment into which *Ac* was inserted (Figure 2B) was cloned and partially sequenced, in a homozygous mutant plant, after inverse PCR (IPCR) amplification with *Ac* oligonucleotides 2 and 6. Oligonucleotides K5 and K3 were designed on each side of the *Ac* insertion point. These oligonucleotides were used to amplify and sequence the insertion point in the heterozygous plant without the 4.5 kb *Ac* band and in a wild-type plant. When the sequence of the mutant was compared with wild-type, a 7 bp footprint, likely caused by the *Ac* excision, was observed in the mutant, thereby creating a mutation in the corresponding gene. This *Ac* footprint generates a stable allele of the *aba2* mutation, called *aba2-s1*.

Progeny from one mutant plant, homozygous for the 4.5 kb *Ac*-hybridizing band, were grown and screened for somatic and germinal reversion events, leading to a normal transpiration rate and survival in greenhouse conditions. Reversion of the mutation to the wild-type phenotype should be correlated with restoration of a wild-type-sized DNA fragment. Four revertant plants (recovered among ~5000 seeds) were analyzed at the molecular level (lines 1–4 in Figure 2C). *Eco*RV digested DNA was blotted and hybridized to the IPCR *Kpn*I fragment (probe C on Figure 2B). Wild-type DNA exhibited a 5.5 kb band. Homozygous *aba2* plants displayed a 10 kb band as expected for a 4.6 kb *Ac* insertion into the 5.5 kb *Eco*RV fragment (line 6 in Figure 2C). The presence of both the 5.5 kb and the 10 kb bands in R_3 revertant plants as in heterozygous R_2 plants (lines 1–5 in Figure 2C) is in accordance with this interpretation. The 2.8 kb band also revealed by this probe, corresponds to an *Eco*RV genomic fragment downstream of the *Ac* insertion point (Figure 2B). Progeny of revertant plants was scored for the early germination phenotype. Revertants 1, 2 and 3 are germinal as they segregated 25% early germination progeny. Revertant 4 gave homogeneous early germination progeny and is therefore a somatic revertant.

ABA2 encodes a 2.5 kb mRNA. Northern blot analysis was performed on total RNA extracted from leaves of wild-type plants and of the *aba2* unstable mutant using probe C (Figure 2B). A single 2.5 kb transcript was observed in wild-type leaves, whereas in mutant leaves mRNA was 7.1 kb long (Figure 2D). This shift of 4.6 kb

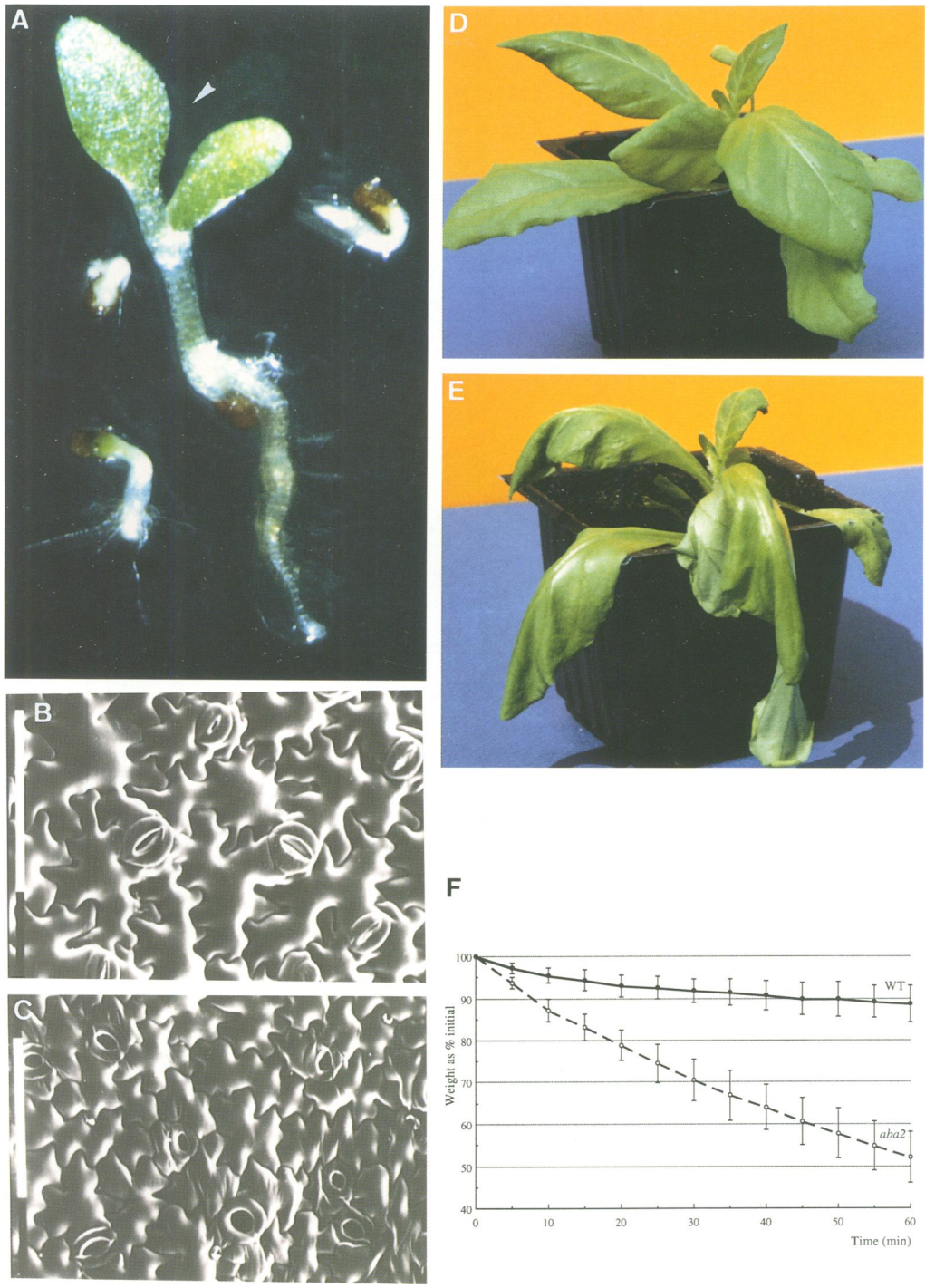


Fig. 1. Phenotype of the *aba2* mutant compared with wild-type *N.plumbaginifolia*. Segregation of the early germination phenotype in the progeny of the mother plant AA67, as observed 10 days after *in vitro* sowing. The arrow points to an *aba2* plant (**A**). Stomata from the abaxial surface of wild-type leaves (**B**) and of mutant leaves (**C**) as seen by scanning electron microscopy. Bars are 0.1 mm long. Phenotype of a homozygous mutant plant in a growth chamber with 90% relative humidity (**D**) and 5 min after the transfer to greenhouse conditions (**E**). Effect of dessication on the weight of wild-type and *aba2* detached leaves (**F**). Leaves were harvested, blotted gently on paper towel and weighed every 5 min in the drying atmosphere of a laminar hood. Mean and standard deviation of six independent experiments are shown.

is consistent with the presence of an *Ac* element into a coding sequence.

The aba2 mutant is impaired in zeaxanthin epoxidation

ABA content is reduced in the aba2-s1 mutant. In order to confirm the ABA deficiency of the mutant, three independent measurements of endogenous ABA and ABA-glucose ester (ABA-GE) levels were performed on leaf extracts of wild-type and of the stable mutant *aba2-s1*. For each measurement, standard error results from five replicates. Wild-type leaves contained 393 ± 57, 444 ± 25 and 1263 ± 36 pmol of ABA per g of dry weight (g DW). The ABA-GE content was respectively 158 ± 11,

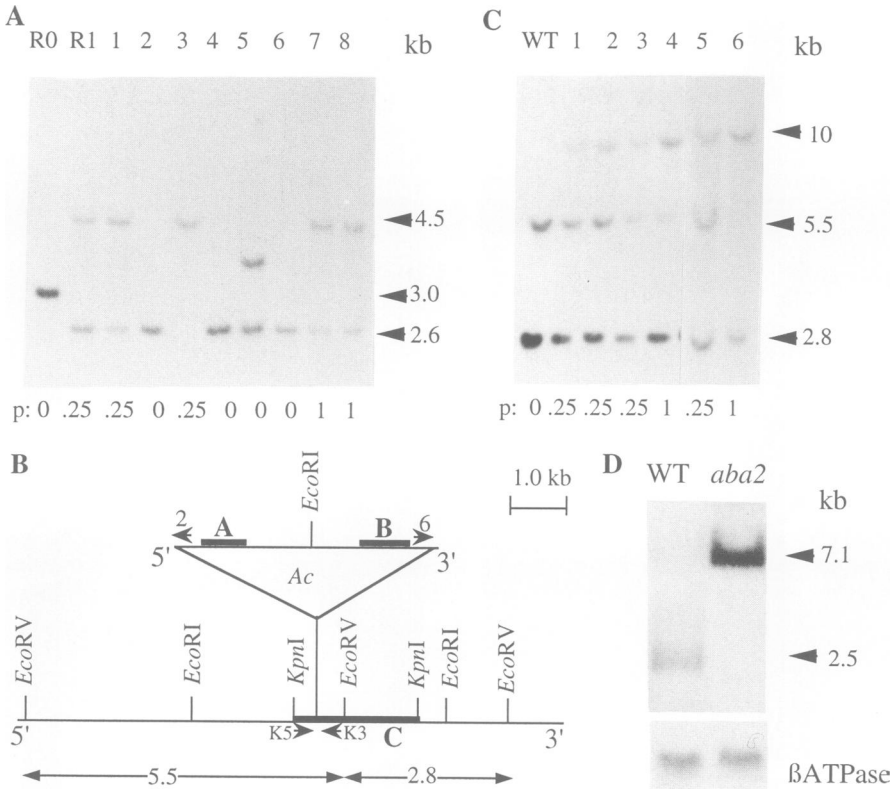


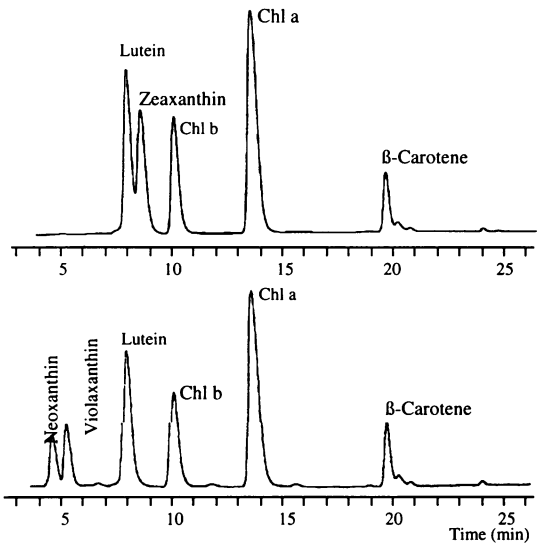
Fig. 2. Molecular analysis of the insertional mutation at the *ABA2* locus. (**A**) Southern blot analysis of *Eco*RI digested DNA from mutant and wild-type progeny of plant AA67, carrying a transposed *Ac* element. R_0 indicates the primary transformant and R_1 indicates the AA67 parental plant. Lanes 1–8 are representative progeny of this plant. 1 and 3 are heterozygous plants segregating 25% mutant progeny; 2, 4, 5 and 6 are wild-type plants; 7 and 8 are two homozygous mutant plants. The blot was hybridized to probe A (in the 5′ border of *Ac*). (**B**) Partial restriction map of the *ABA2* locus. The positions of the probes corresponding to *Ac* and to the flanking DNA are indicated by thick bars A, B and C. Oligonucleotides are indicated by small arrows. The wild-type *Eco*RV fragments detected with probe C are also indicated. (**C**) Southern blot analysis of revertant plants. The digestion was performed by *Eco*RV and hybridization with the IPCR probe C. Lanes are as follows: WT, wild-type; 1–4, revertant progeny plants of a homozygous R_2 mutant plant; 5, a heterozygous progeny of the mother plant AA67; 6, homozygous mutant plant. For panels A and C, p indicates the proportion of mutant siblings in the progeny of each plant analyzed by Southern hybridization. (**D**) RNA gel blot analysis of wild-type and mutant leaves hybridized to the IPCR probe C. A control hybridization with the 1.6 kb *Eco*RI–*Sal*I cDNA fragment of the nuclear-encoded β subunit of *N.plumbaginifolia* mitochondrial ATPase (Boutry and Chua, 1985) is also shown.

163 ± 28 and 236 ± 21 pmol of equivalents of ABA per g DW. The ABA content in the *aba2-s1* mutant was between 23 and 48% of the wild-type ABA content. ABA-GE ranged from undetectable in two of the measurements to 139 pmol of equivalents of ABA per g DW in one of the samples.

Carotenoid composition is modified in the aba2-s1 mutant. The possibility that the *aba2-s1* mutation affects the C_{40} part of the ABA biosynthetic pathway was investigated by analyzing the carotenoid content of both wild-type and mutant leaves (Figure 3A). In three independent measurements, there were no significant differences in the total amount of carotenoids in both genotypes (3065 ± 160 and 3704 ± 900 μg of total carotenoids per g FW respectively). However, there were significant quantitative differences in zeaxanthin and its derived products. Zeaxanthin was undetectable in the wild-type leaves, but accumulated up to 7% of the total pigment content in the mutant leaves. In contrast, violaxanthin, antheraxanthin and neoxanthin were undetectable in mutant leaves, whereas the amount of the three epoxy-carotenoids represented 4.1% of the total carotenoid content in wild-type leaves. Based on these data, it was concluded that the *aba2* mutant is impaired in the epoxidation of zeaxanthin, leading to the accumulation of zeaxanthin and the absence of the following products of the ABA biosynthetic pathway (Figure 3B). *aba2* might therefore be a *N.plumbaginifolia* equivalent of the *A.thaliana aba* mutant (Koornneef *et al.*, 1982), which is blocked at the same biosynthetic step (Duckham *et al.*, 1991; Rock and Zeevaart, 1991).

The xanthophyll cycle consists in de-epoxidation of violaxanthin to zeaxanthin in chloroplasts of plant leaves and algae upon strong illumination and reconversion of zeaxanthin to violaxanthin under non-excessive light levels. Zeaxanthin accumulates during exposure of leaves to high photon flux densities and plays a role in increased chlorophyll fluorescence quenching and protection of the photosynthetic apparatus (reviewed by Demmig-Adams and Adams, 1992). In order to test whether the altered carotenoid profile of the mutant *aba2* had an effect on quantum yield of photosystem (PS) II photochemistry, we measured the chlorophyll fluorescence yield, (*F*m–*F*o)/*F*m, in wild-type and *aba2* rosette leaves. Mean values and standard errors of three independent experiments are 0.81 ± 0.01 for wild-type and 0.77 ± 0.01 for *aba2*, suggesting a faint but reproducible effect of the mutation on the chlorophyll fluorescence yield of PS II. We performed the same measurements with *Arabidopsis aba* and

A



B

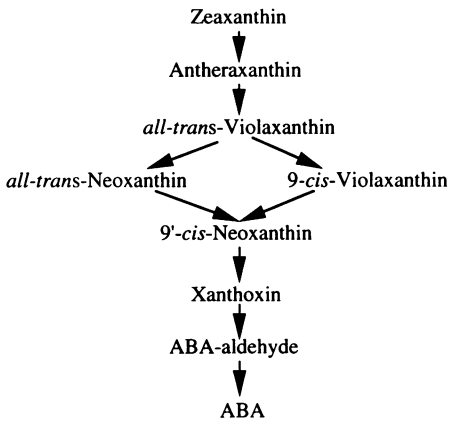


Fig. 3. Carotenoid content analysis. (**A**) Carotenoid profile of one of three independent measurements performed in leaves of wild-type and *aba2-s1* mutant. (**B**) Biosynthesis of ABA from zeaxanthin. Adapted from the pathway proposed by Li and Walton (1990) and Parry *et al.* (1990).

wild-type Landsberg *erecta* plants and found similar results (0.75 ± 0.02 for the mutant and 0.78 ± 0.01 for the wild-type).

Sequence analysis of ABA2 cDNA

A λgt11 cDNA library was screened with the DNA genomic *Kpn*I fragment flanking the *Ac* insertion (probe C on Figure 2B). One positive clone showed an insert of 2.4 kb, which was the expected size for the *ABA2* cDNA as revealed by Northern blot analysis (Figure 2D). The insert was completely sequenced on both strands. This sequence of 2400 bp and the largest open reading frame (ORF) starting with a methionine are shown in Figure 4A. This ORF encodes a polypeptide of 663 amino acid residues, with a predicted molecular mass of 72.54 kDa and an isoelectric point of 7.28. An in-frame stop codon at nucleotide position –20 confirms that the ORF shown in Figure 4A is the entire coding region of the corresponding gene. If the ATG at nucleotide 1 is the initiation codon, the cDNA has 40 bp of 5′ untranslated sequence, a 1989 bp coding region and a 313 bp untranslated 3′ sequence, followed by a poly(A) tail (58 bp long). The untranslated 3′ sequence contains three putative poly(A) addition signals (ATAAA) (Joshi, 1987) starting at nucleotide positions 2015, 2109 and 2276. As indicated by partial sequencing of the *Kpn*I IPCR cloned fragment, the insertion of *Ac* took place into the ORF of the tagged locus, at nucleotide 434 (amino acid 145). The 8 bp duplicated upon *Ac* insertion (Fedoroff, 1989) at *ABA2* locus are shown bold in Figure 4A. A Southern blot analysis of genomic DNA, digested with different restriction enzymes, was performed with the cloned cDNA as a probe. Both at high and low stringency hybridization and wash conditions the gene appeared to exist as a single copy in the genome of *N.plumbaginifolia* (data not shown).

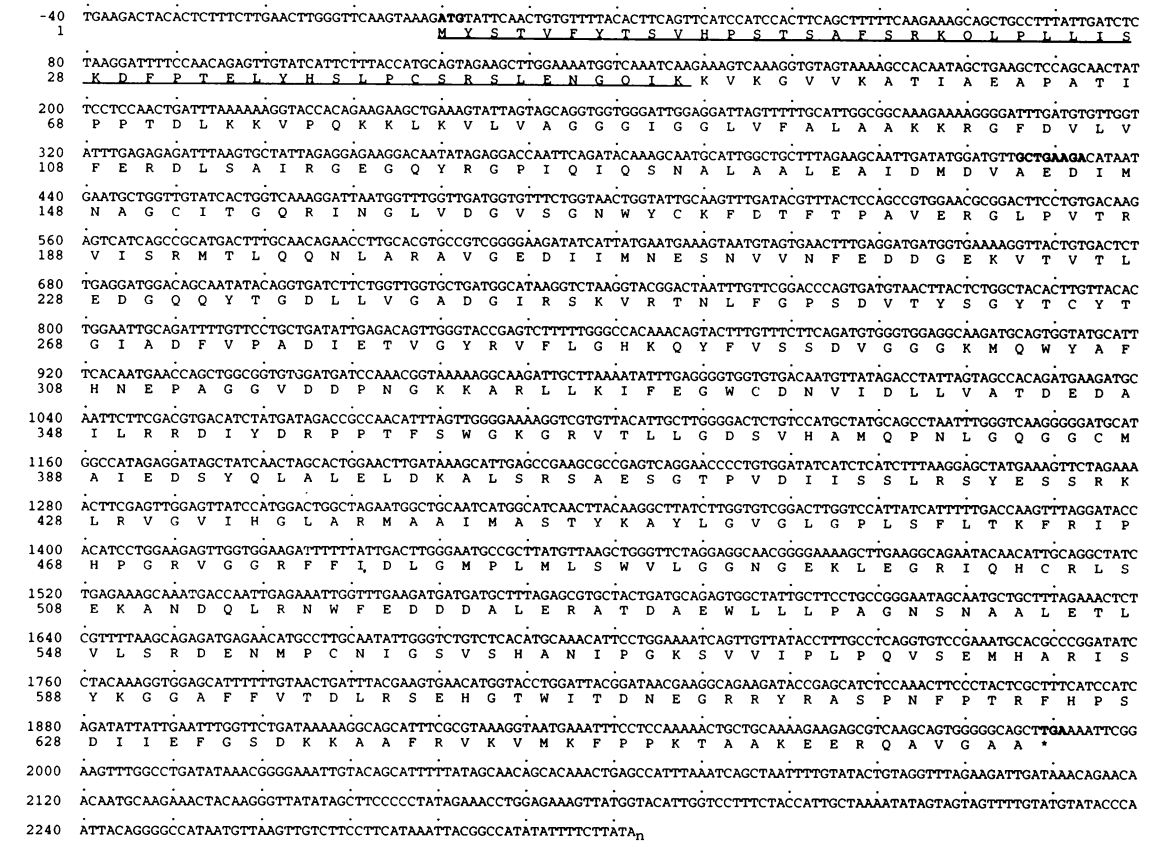
The predicted amino acid sequence of ABA2 protein was compared using the Blast program (Altschul *et al.*, 1990). This search revealed three segments of significant similarity between ABA2 and known *Pseudomonas* sp. and *Escherichia coli* proteins (Figure 4B). The best fits were obtained with NAHG (salicylate 1-hydroxylase; You *et al.*, 1991), UBIH (2-octaprenyl-6-methoxyphenol monooxygenase; Nakahigashi *et al.*, 1992), VISA (ferrochelatase; Nakahigashi *et al.*, 1992), PHEA (phenol monooxygenase; Nurk *et al.*, 1991), PCPB (pentachlorophenol-4-mono-oxygenase; Orser *et al.*, 1993) and PHBH (*p*-hydroxybenzoate hydroxylase; Weijer *et al.*, 1982).

All these protein sequences share a common function, which is hydroxylation or oxidation of an aromatic ring. This is in agreement with the presumed activity of the ABA2 protein, i.e. the epoxidation of zeaxanthin, a substrate containing a cyclohexane ring. These enzymes use a common cofactor, either FAD or NAD(P). Accordingly, the amino acid residues 81–109 of ABA2 fit the consensus sequence (fingerprint) involved in ADP binding (Figure 4B). This glycine-rich motif, common to cofactor-binding enzymes, predicts whether a particular amino acid sequence can fold into a βαβ-unit with binding properties for the ADP moiety of NAD(P) or FAD (Wierenga *et al.*, 1986). In addition, a putative FAD-binding site similar to the amino acid sequence MQHGRLFLAGD, which is involved in FAD binding of PHBH (Wijnands *et al.*, 1986), was identified. The ABA2 residues 359–372 show nine matches with this sequence, enough to guarantee the conformation which is necessary for FAD binding (Figure 4B). Consistent with this conclusion, a few residues after this stretch (amino acid positions 379 and 382) which are identical to those found in both the PHBH and NAHG sequences, have been described as having structural and functional importance for FAD-binding activity (You *et al.*, 1991). A third region of similarity between the ABA2 and the prokaryotic amino acid sequences is comprised between amino acid residues 230 and 249 of ABA2. This sequence has not been described to date, but is present in all the amino acid sequences showing some similarity with ABA2.

ABA2 is imported into the chloroplast

The database searches indicated the existence of an amino terminal extension in the cDNA product in comparison with the *Pseudomonas* and *E.coli* proteins. The extension was located between amino acid residues 1 and 50, and

A



B

a

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ADP-binding βαβ fold   xy-y-G-G--G---y--y (loop) y-y-z
ABA2 (aa 81-109)       KVLVAGGGIGGLVFALAA KKRGFD VLVFE
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b

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   PCPB     TISPRWVI GADGVRSRVRE (185)
   VISA     MLTARLVI GADGANSWLRN (171)
   UBIH     TLTGRVLV AADGTHSALAT (168)
   PHEA     TIRAKYLI GADGARSKVAA (116)
   NAHG     EYRCDLLI GADGIKSALRS (170)
   ABA2     QYTGDLLV GADGIRSKVRT (249)
consensus   --x---yy GADG--S-yR-
                    A       A
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c

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FAD binding site consensus        T/M ----ywyyGD
PHBH FAD binding (aa 277-287)     M   QHGRLFLAGD
NAHG FAD binding (aa 304-314)     Y   VHGRVVLIGD
ABA2 (aa 359-372)                 TFSWGKGRVTLLGD
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Fig. 4. Sequence analysis of the cloned cDNA. (**A**) Complete cDNA sequence together with the deduced amino acid sequence for the largest ORF starting with a methionine. The first initiation codon and the stop codon are indicated in boldface, as well as the duplicated sequence in the genomic locus after the insertion of *Ac*. The chloroplast signal peptide is underlined. (**B**) Three domains of significant similarity with known proteins are shown: the ADP-binding motif (a), the central motif of unknown function (b) and the FAD-binding motif (c). Amino acid positions are indicated in parentheses. Abbreviations: (-) any residue; (w) Y, W or F (amino acids with an aromatic group); (x) K, R, H, S, T, Q or N (basic or hydrophilic amino acids); (y) A, I, L, V, M or C (small and hydrophobic amino acids); (z) D or E (acidic amino acids).

appears underlined in Figure 4A. The amino acid content in this region revealed a low level of acidic amino acids and an enrichment in serine and threonine. These amino acids constitute respectively 18 and 8% of the total amino acid content in this stretch, whereas they are much less abundant in the complete ABA2 sequence (6.8 and 4.9% respectively). This characteristic has been described in known chloroplast targeted proteins as being involved in the import of nuclear proteins into this organelle (Von Heijne *et al.*, 1989).

In order to investigate the chloroplastic localization of the ABA2 protein, we performed *in vitro* transcription and translation of the corresponding cDNA clone. The labeled translation products were incubated with purified pea chloroplasts. An aliquot of the reaction was treated with thermolysin to digest proteins on the surface of the chloroplast, so only the proteins inside the organelles should remain intact. Figure 5 shows the fluorography of an SDS–polyacrylamide gel loaded with the chloroplast importation reaction of ABA2 protein. PSAD, a 28 kDa PS I protein, used as a positive control, was imported into pea chloroplasts and processed into a mature 20 kDa protein as expected (data not shown). The ABA2 protein (72.5 kDa) was also imported to the chloroplast and processed into a 67 kDa mature protein (Figure 5), in accordance with sequence data analysis.

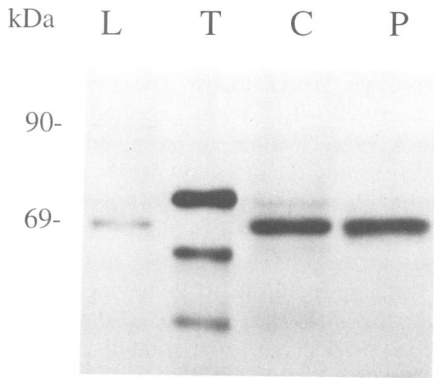

Fig. 5. Chloroplast import study of *ABA2* translation product. Lane L is loaded with the molecular weight markers, T is loaded with 1 µl of the 50 µl translation mix, C is loaded with the import reaction with chloroplasts (corresponding to 1/4 of the total import reaction) and P is loaded with the protease treated import reaction (corresponding to 1/4 of the total import reaction).

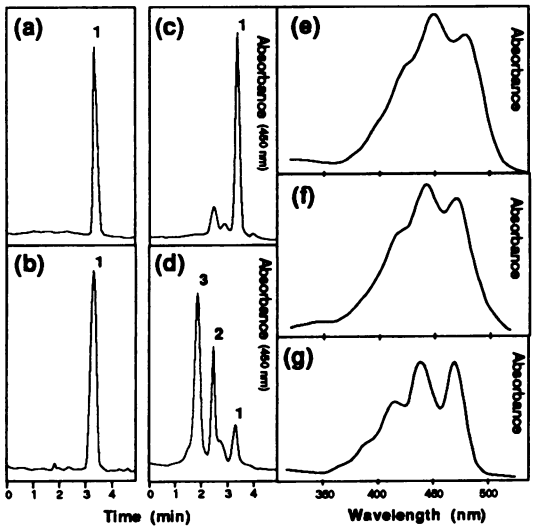

Fig. 6. *In vitro* zeaxanthin epoxidase activity of *E.coli* extracts expressing the *ABA2* cDNA, as visualized by HPLC fractionation of the reaction mix and absorption spectra of the pigments. Enzymatic assays were performed with a bacterial strain containing a control plasmid pQE31 without insert, in absence (**a**) or presence (**b**) of *C.annuum* stroma proteins, or with a bacterial strain containing the plasmid pQE31-*ABA2* cDNA, in the absence (**c**) or presence (**d**) of stroma protein extract. Typical absorption spectra of peaks 1 (zeaxanthin) (**e**), 2 (antheraxanthin) (**f**) and 3 (violaxanthin) (**g**) are presented.

Zeaxanthin epoxidation activity of ABA2 protein

ABA2 cDNA coding sequence was expressed in an *E.coli* strain pAPU211R producing zeaxanthin, after subcloning in pWSK129 vector (Wang and Kushner, 1991) but no *in vivo* synthesis of epoxy-carotenoid products was detected in cell extracts (data not shown). Therefore zeaxanthin epoxidase activity was assayed *in vitro* after addition of a stroma protein extract of *Capsicum annuum* in order to provide a putative factor necessary for protein activity. *ABA2* cDNA was cloned in pQE31 vector as described in Materials and methods and the bacterial protein extract was incubated with zeaxanthin. Significant amounts of reaction products (antheraxanthin and violaxanthin) were detected only when a stroma protein extract was added (Figure 6). In particular, no synthesis of xanthophyll products was observed if the stroma protein extract was added to the bacterial extract when no *ABA2* cDNA insert was present. These data show firstly that ABA2 protein catalyses the conversion of zeaxanthin into antheraxanthin and subsequently violaxanthin. Secondly, an unindentified factor present in chloroplast extract is necessary for the activity of the protein. This factor could be a protease cleaving the transit peptide present in the constructs we used, or a cofactor, absent in *E.coli*, necessary for protein activity or assembly. Further work is in progress to delineate this phenomenon.

ABA2 cDNA complements the aba2-s1 mutation

Leaf disks of *aba2-s1* plants were transformed via *Agrobacterium tumefaciens* with two different constructs, one containing 35S-*ABA2* ,cDNA and the second no *ABA2* cDNA insert (control plasmid). Primary transformants were tested for their carotenoid composition. As expected, regenerants obtained after transformation with the control plasmid showed an absence of epoxy-carotenoids and an accumulation of zeaxanthin, as in the untransformed plants. Regenerants obtained after transformation with the 35S-*ABA2* cDNA construct showed a wild-type transpiration rate and carotenoid complement (data not shown). Thus the cloned cDNA was able to restore the wild-type phenotype when introduced in a stable mutant background, providing further evidence for the tagging of the epoxidase gene.

Nicotiana plumbaginifolia ABA2 and A.thaliana ABA loci are homologous

Comparison of the *ABA2* cDNA sequence with the *Arabidopsis* EST sequence database led to the identification of a homologous EST (accession number T45502). This EST sequence is 480 bp long and its predicted ORF between base pairs 4 and 210 shows 71% identity and 81% similarity with the *N.plumbaginifolia* ABA2 amino acid sequence. Oligonucleotides AT3 and AT4 were designed, based on the T45502 sequence, and used to screen the *Arabidopsis* CIC YAC library (Creusot *et al.*, 1995). The PCR primers identify a unique YAC clone (CIC3B1) located at the bottom of chromosome five, part of a YAC contig containing the RFLP marker ve028 [map position 134.1 (Lister, 1995); D.Bouchez, personal communication]. This location correlates exactly with the map position of the *ABA* locus of *Arabidopsis* reported by Koornneef *et al.* (1982). This result, together with the phenotypical analogy of the mutants, suggests that the *ABA2* and the *ABA* loci are homologous. Complementation experiments of *Arabidopsis aba* mutation were therefore performed to have a definite proof of this homology.

Wild-type *Arabidopsis* plants were transformed with the 35S-*ABA2* construct previously described. Pollen of primary transformants was then used to pollinate the *aba* mutant A26 (Koornneef *et al.*, 1982). Kanamycin-resistant siblings, heterozygous for the *aba* mutation (*ABA/aba*, T-DNA/–), were selected. Kanamycin-resistant selfed progeny of one plant were then tested for carotenoid content. Presence of violaxanthin was detected in all the 35 plantlets tested. This 35:0 ratio was expected if *ABA2* cDNA

was complementing *aba* mutation and was significantly different from the otherwise expected ratio (26:9). Two independent T-DNA loci were segregating in this progeny, as deduced from the ratio of kanamycin-resistant to kanamycin-sensitive plants (267:22). An eventual physical linkage between a T-DNA insertion site and the *ABA* locus would lead to a mutual exclusion of the kanamycin resistance trait and *aba/aba* genotype. The presence of two unlinked insertions rules out this possibility. In this case, only one of the T-DNA insertions could be possibly linked to the *ABA* locus and, among 35 kanamycin-resistant plants tested, the expected ratio of violaxanthin-producing plants to non-producing plants would be 28:7. Finally, Northern analysis was performed on *A.thaliana* wild-type (ecotype Landsberg) and *aba* mutant A26 with the PCR fragment amplified with the AT3-AT4 primers. A 2.7 kb mRNA was detected in wild-type leaf extract. No band, or a very faint hybridizing band, was observed in mutant A26 (data not shown) suggesting that the mutation affects transcription or RNA maturation of the *ABA* gene. Mapping of *Arabidopsis* EST T45502 at the *ABA* locus, complementation of *aba* mutation by the *N.plumbaginifolia ABA2* cDNA and reduced transcript accumulation in *aba* mutant A26 demonstrate that the *ABA2* locus of *N.plumbaginifolia* and the *ABA* locus of *A.thaliana* are homologous.

Discussion

We have isolated, by insertional mutagenesis, a gene involved in ABA biosynthesis, which has been called *ABA2*. ABA-deficient mutant *aba2* was identified by its early germination phenotype. Screening for early germination is possible only for species like *N.plumbaginifolia*, the seeds of which exhibit a strong primary dormancy. Freshly harvested seeds can germinate only under alternate temperature conditions or after gibberellin treatment. In plant species producing seeds with a low primary dormancy, as is the case for *Arabidopsis*, screening for ABA-deficient mutants at the seed level can be achieved only after blocking gibberellin biosynthesis either by mutations or by inhibitors (Koornneef *et al.*, 1982). The *ABA2* locus is the second locus affecting ABA biosynthesis identified in *N.plumbaginifolia*. As with *aba1* and ABA-deficient mutants in other species (reviewed by Giraudat *et al.*, 1994), *aba2* is characterized by a high tendency to wilt, restored by ABA application, inability to close stomata and reduced primary dormancy, confirming the crucial role of ABA in water stress tolerance and in seed dormancy.

Cloning of the *ABA2* gene was performed after tagging by the autonomous *Ac* element of maize. This is the first report of the isolation of a transposable element-tagged gene in *N.plumbaginifolia*. Since the report of Chuck *et al.* (1993) on the tagging of a petunia flower color gene by *Ac*, several *Ac*-, *Ds*- or *Spm*-tagged genes have been isolated in five different heterologous plant species. The success reported in *N.plumbaginifolia* further supports the value of the transposable element-tagging strategy in the isolation of new plant genes in many different species, in contrast with the T-DNA tagging approach, a powerful strategy, but mainly restricted to *Arabidopsis*.

We took advantage of the tagging of the *ABA2* locus by an autonomous transposable element to recover a few revertant siblings in the progeny of a homozygous mutant plant. The four revertants recovered all exhibited an *Ac* excision from the *ABA2* locus. The low frequency of reversion observed (~0.1%) was expected because the *Ac* insertion took place within an exon, so only excision events re-establishing the original ORF of the tagged gene would permit the reversion to wild-type phenotype. As *Ac* excision is often imprecise and leaves a few base pairs at the donor locus (Fedoroff, 1989), only those events leaving 0, 3 or 6 bp footprints while having no stop codon, would eventually allow the reversion of the mutation. One of the revertants appeared to be somatic. Such reversion of unstable *aba2* mutation cannot be distinguished from germinal reversion in terms of phenotype, because ABA is not a cell-autonomous molecule. There is substantial evidence that ABA can be translocated in the plant, acting as a chemical signal in root-to-shoot communication (Davies *et al.*, 1994) as well as between different cell compartments (reviewed by Zeevaart and Creelman, 1988). ABA translocation from root to shoot was demonstrated by grafting *aba2* mutant plants on *Nicotiana tabacum* stocks which subsequently restored the wild-type phenotype.

The *aba2* mutant of *N.plumbaginifolia* is impaired in ABA biosynthesis as a result of a lesion in the C_{40} part of the ABA biosynthetic pathway. The carotenoid content in leaves of the mutant was consistent with a mutation impairing the zeaxanthin epoxidase activity. The presence of ABA (23–48% of the wild-type content) and ABA-GE in *aba2-s1* leaves was unexpected. The *aba2-s1* mutant allele is stable due to a 7 bp footprint left after *Ac* excision. The stop codon created in the excision footprint would generate a truncated protein, at the amino acid position 146 of the original *ABA2* ORF, which is presumed to be inactive. Moreover, our data indicate that the *ABA2* locus exists as a single copy in the *N.plumbaginifolia* genome. Our results suggest at least three hypotheses to explain the production of ABA in *aba2-s1* plants. First, a completely different pathway, like the C15 pathway described in fungi (Zeevaart and Creelman, 1988), might exist in higher plants. Nevertheless, this hypothesis is not supported by genetic data, as all the mutants described so far are affected in the C40 pathway (Taylor, 1991). Secondly, a residual synthesis of ABA2 protein might occur despite the presence of a stop codon (Valle and Morch, 1988). Experiments are underway to seek for crossreacting material with antibodies. Finally, the epoxidation of zeaxanthin, when accumulated, could be achieved at low rate by a less specific enzyme. This kind of situation has already been described in the carotenoid biosynthesis pathway. The capsanthin/capsorubin synthase, which is involved in the synthesis of the species-specific red carotenoids in *C.annuum* fruits, has been shown to exhibit lycopene β-cyclase activity when expressed in *E.coli* (Hugueney *et al.*, 1995). Considering the essential role of ABA in plant development, the existence of secondary biosynthetic pathways, like the shunt pathway from ABA-aldehyde to ABA (Rock *et al.*, 1991), would be of physiological importance and explain why only ABA-deficient leaky mutants have been isolated to date. It is interesting to note that the ABA level reported for *aba* mutant A26 leaves was 17% of the wild-type ABA content (Koornneef *et al.*, 1982) and that, in stress conditions, this

mutant synthesized *de novo* only 2.7% of the ABA synthesized by the wild-type (Rock and Zeevaart, 1991). This explains why *Arabidopsis aba* and *N.plumbaginifolia aba2* mutants, despite their relatively high level of accumulated ABA, are unable to overcome hydric stress by newly synthesized ABA.

The biochemical lesion of mutant *aba2* suggested that ABA2 protein was involved in epoxidation of zeaxanthin. Identified domains of ABA2 protein by sequence analysis, in particular the existence of an ADP-binding motif and an FAD-binding motif, were in agreement with an epoxidase activity. Moreover, all the prokaryotic proteins which shared similarity to ABA2 have an oxidative or hydroxydative activity on an aromatic substrate and, in accordance, zeaxanthin contains a cyclohexane ring. Zeaxanthin epoxidase activity of *E.coli* extracts expressing ABA2 protein gave a definite proof of the function of this protein. Furthermore, zeaxanthin epoxidase is shown here to convert zeaxanthin into antheraxanthin and violaxanthin, as previously hypothesized. Because of the symmetry of zeaxanthin, a unique enzyme was supposed to catalyze the formation of antheraxanthin and its subsequent conversion to violaxanthin (Taylor, 1991).

The apo-carotenoid zeaxanthin, as well as the products of the epoxidation reaction (antheraxanthin and violaxanthin), are found in chloroplast membranes. Moreover, zeaxanthin epoxidase was identified in the chloroplast envelope of *Spinacia oleracea* leaves by Costes *et al.* (1979) and in the major light-harvesting pigment–protein complex in *Secale cereale* leaves (Gruszecki and Krupa, 1993). In accordance, ABA2 protein has been shown here to be chloroplast imported. In agreement with the report of Rock and Zeevaart (1991), our data imply that the same epoxidase is involved in the xanthophyll cycle and in epoxy-carotenoid biosynthesis leading to ABA formation. The epoxidase enzyme would then be simultaneously associated with the envelope and with the thylakoid membranes. This hypothesis could be tested by immunolocalization of the protein.

When light energy input exceeds the capacity for energy utilization through photosynthetic electron transport, the xanthophyll cycle is activated and zeaxanthin is accumulated in the thylakoids. The zeaxanthin accumulation constitutes the major protective process of the photosynthetic apparatus, because of its role in dissipation of excess energy by quenching of the chlorophyll fluorescence (Demmig-Adams and Adams, 1992). Rock *et al.* (1992) reported that the zeaxanthin accumulation and epoxy-carotenoid deficiency in the *aba* mutant of *Arabidopsis* have little effect on photochemical activity of PS II, whereas they strongly affect the thylakoid stacking. We showed that the quantum efficiency of PS II in the *aba2* mutant of *N.plumbaginifolia* and in the *aba* mutant of *A.thaliana* is only slightly reduced, in accordance with Rock *et al.* (1992). Our results indicate that *N.plumbaginifolia* and *Arabidopsis* plants expressing the *ABA2* gene under a strong constitutive promoter exhibit a wild-type phenotype and carotenoid complement (data not shown). Further experiments have to be performed to investigate the effect of a deregulation of zeaxanthin epoxidation on xanthophyll accumulation and activity of photosynthetic apparatus. Moreover, measurements of ABA levels in these plants will indicate if zeaxanthin epoxidation is a rate-limiting step of ABA biosynthesis which seems unlikely since xanthophylls are abundant in plants compared with ABA (Li and Walton, 1990).

In conclusion, ABA plays an important role in various physiological processes such as resistance to stresses, seed development and dormancy. Nevertheless correlation between ABA levels and its physiological effects is often unclear. Isolation of the *ABA2* locus constitutes the first tool for a better understanding of regulation and location of ABA biosynthesis, and of its role in plant physiology. It also offers the opportunity to study the effects of modification of ABA synthesis in specific tissues and at various developmental stages in transgenic plants. Zeaxanthin epoxidase is also involved in the xanthophyll cycle, together with a yet unidentified de-epoxidase which converts violaxanthin into zeaxanthin (Demmig-Adams and Adams, 1992). Study of *ABA2* gene expression and its modification will allow us to study the regulation of the xanthophyll cycle and the role of xanthophylls in photosynthesis.

Materials and methods

Plant material and growth conditions

Transgenic plants of *N.plumbaginifolia* cv. *viviani* carrying the *Ac* element were obtained and characterized previously (Marion-Poll *et al.*, 1993). Screening for mutant phenotypes was performed as described (Marion-Poll *et al.*, 1993). After *in vitro* germination of seeds (25/17°C, 6 h photoperiod), young plantlets were transferred to soil. Mutant *aba2* plantlets were unable to grow under standard greenhouse conditions and were grown in a growth chamber with 70–90% relative humidity, 16 h photoperiod and 25/17°C. All molecular and biochemical analyses were performed on unstressed leaves harvested before the flowering period. The water stress protocol used to determine the rate of water loss has been described by Parry *et al.* (1991). Plants of *A.thaliana* wild-type (ecotype WS or ecotype Landsberg) and ABA-deficient mutant A26 (ecotype Landsberg) were grown in greenhouse conditions.

Southern and Northern analysis

Genomic DNA extraction from 2 g of leaf material and Southern analysis were performed as described previously (Marion-Poll *et al.*, 1993), using probes A (0.7 kb *Acc*I–*Hin*dIII fragment of 5′ *Ac* extremity), B (0.8 kb *Hin*dIII–*Acc*I fragment of 3′ *Ac* border) and C (an IPCR probe described below), as shown in Figure 2B.

Total leaf RNAs were extracted according to Verwoerd *et al.* (1989) and 8 μg were loaded on a 1.2% agarose gel containing formaldehyde (Sambrook *et al.*, 1989). Northern blots were performed under stringent conditions using probe C (Figure 2B).

Inverse PCR

IPCR amplification of *Ac* flanking sequences was performed as described by Earp *et al.* (1990). Genomic DNA of a plant exhibiting a unique *Ac* insertion linked to the *aba2* mutation was digested by *Kpn*I and ligated with T4 DNA ligase. The PCR reaction was performed with *Ac* oligonucleotides # 2 (5′-CGATAACGGTCGGTACGGGA-3′) and # 6 (5′-CCCGTTCGTTTTCGTTACCG-3′), depicted in Figure 2B. The oligonucleotide # 2 hybridizes 44 bp away from the 5′ *Ac* border. The oligonucleotide # 6 hybridizes 115 bp away from the 3′ *Ac* extremity. Amplification was achieved with a cycle of 45 s denaturation at 94°C, 30 s annealing at 60°C and 2 min polymerase extension at 72°C, repeated 30 times, with a final 10 min 72°C extension in a Perkin Elmer Cetus DNA thermal cycler PEC480.

The 2 kb long amplified fragment was cloned and used as probe C (Figure 3B) to hybridize Northern blots, Southern blots and to screen a cDNA library. Oligonucleotides K3 (5′-TATCAAAGCTTATGTGT-GATTG-3′) and K5 (5′-TTGGCGGCAAAGAAAAGGGG-3′) were designed on each side of the *Ac* insertion point to facilitate the sequencing of excision footprints. PCR conditions were similar to those described earlier, but the annealing temperature was 56°C instead of 60°C.

Biochemistry

Analysis of ABA and its glucose ester was performed as described by Julliard *et al.* (1993). The hormonal levels were measured in non-stressed leaves pooled from three to five plants grown in a 70–90% relative humidity growth chamber. Leaf samples were immediately frozen in liquid nitrogen and lyophilized prior to grinding into powder. For extractions, 15 mg DW, corresponding to 0.3 g FW, were used. Extraction in non-oxidative methanol:water (80:20, v/v), pre-purification and high performance liquid chromatography (HPLC) fractionation using a 0.2% formic acid:methanol gradient were performed as described (Julliard *et al.*, 1993). Quantitation of immunoreactive molecules was performed using an enzyme-linked immunosorbent assay (ELISA). This ELISA was slightly modified, using a monoclonal anti-ABA antibody purchased from Phytoscience (Angers, France), labeled with peroxidase conjugated goat antibody to mouse immunoglobulins (Sigma).

For chlorophyll and carotenoid measurements, leaf samples (100 mg FW) were frozen in liquid nitrogen prior to grinding in a mortar with liquid nitrogen and ~5 mg $NaHCO_3$. The pigments were extracted with an acetone:methanol:ether petrol mixture (50:30:20, by vol). Extracts were centrifuged for 5 min at 12 000 *g* and the supernatant stored on ice. The pellet was resuspended in the above solvent and centrifuged again. This procedure was repeated until the supernatant was colourless. Final supernatants were passed through 0.5 μm filters and evaporated. The dry extracts were stored at –20°C in darkness after bubbling with argon. Carotenoid pigments were separated by non-aqueous reversed phase HPLC as described by Gilmore and Yamamoto (1991).

Chlorophyll fluorescence measurements

In vivo chlorophyll fluorescence was measured with a PAM-101 fluorometer (Walz, Effeltrich, Germany) according to Havaux and Davaud (1994). The maximal quantum yield of PS II photochemistry was estimated in dark-adapted leaves as (*Fm*–*Fo*)/*Fm*, where *Fo* is the initial level of chlorophyll fluorescence and *Fm* is the maximal fluorescence level induced by an 800-ms flash of intense white light.

cDNA library construction and screening

Young seedlings at the first leaf stage (3–4 weeks old) were harvested (including roots) and total RNA was purified. The poly(A)$^+$ fraction was selected over oligo(dT) spin columns (Pharmacia). Double-stranded cDNA was synthesized using a cDNA synthesis kit (Promega) and oligo(dT)-*Not*I primer–adaptor. After the ligation of *Eco*RI adaptors (Pharmacia), the cDNA was digested with *Not*I and directionally cloned into the λgt11 *Sfi*–*Not* vector (Promega). About 1.5×10^6 independent recombinants were obtained after phage packaging (Stratagene) and infection in the strain LE 392 (Promega).

The λgt11 cDNA library was amplified in the *E.coli* strain RY 1090 (Promega). Phage plaque hybridization with probe C was performed according to standard procedures (Sambrook *et al.*, 1989). Washes were done under low stringency conditions in 2× SCC, 0.5% sarkosyl at 65°C. Four positive recombinant phages were obtained after screening of 3×10^5 plaques. Prior to cloning, their size was analysed by a PCR with λgt11 sequencing primers (Promega) with the amplification conditions given by the manufacturer. The insert of one of these phages showed the same size as the transcript detected in Northern analysis and was subsequently cloned in the *Eco*RI–*Not*I sites of a pBS-SK$^+$ vector (Stratagene) and in the *Eco*RI–*Sac*I sites of a pGEM-7Z vector (Promega). These allowed the sequencing of both strands by partial digestion with an Exonuclease III deletion kit (Pharmacia). The nucleotide sequence was determined on double-stranded DNA using the PRISM Ready Reaction kit (Applied Biosystems) following the instructions of the manufacturer and using a Perkin Elmer Cetus DNA thermal cycler PEC480 and an ABI 373A sequencer (Applied Biosystems).

Chloroplast import study

The *ABA2* cDNA cloned into a pBS-SK$^+$ vector was translated from the T3 RNA polymerase promoter. The *PSAD* contruct, encoding a chloroplast localized PS I protein (Kjarulff and Okkels, 1993) was used as a positive control for import reactions. *In vitro* transcription–translation was performed with 1 μg of each plasmid and with ^{35}S-labeled methionine using the TNT Coupled Reticulocyte Lysate System (Promega). Chloroplasts were isolated from leaves of 2-week-old pea plants according to Cline *et al.* (1985). The import assays reactions and the sample analysis on SDS–polyacrylamide gels (12% for PSAD import reaction and 5% for ABA2 import reaction) were done as described by Hoff *et al.* (1995).

ABA2 protein expression in E.coli

Cloned *ABA2* cDNA was amplified by PCR using a 5′ primer (5′-CGCG-GATCCGTATTCAACTGTGTTTTACACTTCAGTTCA-3′) which contains a *Bam*HI site and removes the ATG codon, and a 3′ primer M13–20 (5′-GTAAAACGACGGCCAGT-3′) of pBS-SK$^+$ vector. PCR product was then cloned in *Bam*HI and *Sac*I sites of pQE31 vector (Qiagen). A histidine-tag encoded by pQE31 vector was added at the N-terminus of the expressed protein. Bacteria producing the ABA2 protein were sonicated in a buffer containing 50 mM Tris–HCl pH 7.6, 1% Tween-20 and 500 μM FAD. Protein extract (2 mg of total protein) was incubated at 25°C in 250 μl of the same buffer containing 50 nM zeaxanthin, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM NADPH and 250 μg of stroma protein of *C.annuum* (Hugueney *et al.*, 1992). The substrate zeaxanthin was extracted from a bacterial extract of an *E.coli* strain expressing the carotenoid biosynthesis genes of *Erwinia herbicola* (Hundle *et al.*, 1994) and dissolved in 1% Tween-80. After 2 h of incubation, xanthophylls were extracted in chloroform:methanol (2:1, v/v), analyzed by HPLC (Nova Pak C18 column) and detected by photodiode array detector.

Mapping of the Arabidopsis EST

PCR screening of the CIC YAC library was performed, as described by Creusot *et al.* (1995), on three-dimension YAC pools. Oligonucleotides AT3 (5′-ATCTCGAGTTTACAAAGACGGAGC -3′) and AT4 (5′-AAA-AGCTTAATTTCACCATCGGTT-3′) were designed based on the T45502 EST sequence to give a PCR product of 230 bp. On genomic DNA, these primers amplify a unique 416 bp DNA fragment with the PCR conditions described previously, and an annealing temperature of 55°C.

Transformation procedure

The plant transformation vector pKYLX71–35S^2 was used to clone *ABA2* cDNA between a 35S promoter with duplicated enhancer domain B and a rbcS 3′ region (Maiti *et al.*, 1993). The resulting plasmid (35S-*ABA2*), together with a control plasmid without the cDNA insert, were introduced by triparental matings into a disarmed LBA4404 strain of *A.tumefaciens*. Leaf disk transformation of *in vitro* cultured homozygous *aba2-s1* plants, and selection of regenerant shoots were performed as described by Marion-Poll *et al.* (1993).

The same *A.tumefaciens* strain containing the plant transformation vector 35S-*ABA2* was used to transform wild-type *A.thaliana* plants (ecotype WS) by vacuum infiltration (Bechtold *et al.*, 1993). Primary transformants were used as pollen donors in crosses with the *aba* mutant (Koornneef *et al.*, 1982). The resulting heterozygous plants were grown to maturity and allowed to self. Leaf carotenoid analysis was performed on their progeny.

Accession number

We submitted the sequence of the zeaxanthin epoxidase gene to the EMBL Nucleotide Sequence Database. The accession number is X95732.

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